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Biochimica et Biophysica Acta 1310 (1996) 201–211

BBA Biochimica et Biophysica Acta

Cell-specific regulation of the stably expressed serotonin 5-HT_{1A} receptor and altered ganglioside synthesis

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Received 7 April 1995; revised 18 August 1995; accepted 18 September 1995

Abstract

Neurotransmission is dependent on the presence of neuronal receptors at the synapses, and important cell surface molecules such as gangliosides are pivotal in the maintenance of synaptic contacts. To study the interrelationship between these two classes of molecules, we achieved stable expression of the hippocampus- and CNS-localized serotonin 1A receptor (5-HT_{1A}-R) in three 5-HT_{1A}-R-deficient neuronal cell lines and also the control, non-neural CHO cells. A strong passage dependence of 5-HT_{1A}-R expression, as measured by mRNA levels as well as membrane binding to the selective agonist [^{3}H]8-OH-DPAT, was observed only in the HN2 (hippocampal) and NCB-20 (CNS) cells which are derived from tissues of natural occurrence of the 5-HT_{1A}-R. A paradigm of stress was obtained by carrying out continuous culture of cells without feeding. During this a dramatic increase in 5-HT_{1A}-R mRNA and [^{3}H]8-OH-DPAT binding was observed only in the neuronal cells after confluence and during decreased cell viability (days 10/11). This was not due to differentiation, since deliberate serum deprivation and differentiation of cells did not result in any dramatic increase in 5-HT_{1A}-R expression. Analysis of ganglioside synthesis by pulse labeling of the transfected cells produced striking results. In the dorsal root ganglion (DRG) derived F-11 cells which show low but significant levels of complex gangliosides before transfection, the mere presence of the serotonin 1A receptor resulted in a dramatic increase in synthesis of gangliosides comigrating with G_{M2}, G_{D1a}, G_{D1b}, and G_{T1b} (20-fold by densitometry). In contrast, there was only a 2-fold increase in the overall content of complex gangliosides in the presence of the 5-HT_{1A}-R. In the NCB-20 cells which contain only G_{D1a} but no G_{D1b} or G_{T1b} before transfection, a decrease in G_{D1a} synthesis was observed following transfection. Also agonist (8-OH-DPAT) binding to the serotonin 1A receptor in NCB-20 cells produced a 3-fold increase in synthesis of a ganglioside comigrating with G_{M3}. Thus, our neuroblastoma transfectants help demonstrate stress-induced regulation of the 5-HT_{1A}-R, which in turn exerts a strong and cell type-specific control over such essential cell-surface determinants like gangliosides.

Keywords: Serotonin 1A; Nutrient deprivation; Stress induction; Neuronal differentiation; Ganglioside

1. Introduction

Behavioral studies with animal models and human subjects have shown strong correlation between receptor-mediated neurotransmission and functional states of several important regions of the brain. Thus, Parkinson's disease has been associated with the loss of dopaminergic cells [1], Huntington's disease with the persistent action of glutamate on the NMDA receptors resulting in the loss of striatal neurons [2], and myasthenia gravis with the loss of cholinergic transmission [3]. The opiate receptors have long been known for their analgesic action [4] which is often associated with addictivity and the adrenergic receptors are understood to be involved in affective disorders [5] as well as in the control of blood pressure [6]. Kandel and coworkers have shown that in *Aplysia*, potentiation of long-term memory by serotonin treatment results in synthesis of new proteins [7]. In recent years, the multiple subclasses of serotonin receptors have received much attention for their involvement in wide ranging behavioral aspects such as sleep disorders, emotional balance, anorexia, sensation of pain such as migraine, and long- and short-term memory [8–11]. Extensive studies are thus required to understand at the molecular level, receptor-mediated control of neurological functions in the multiple serotonergic pathways. One effective way of initiating

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SSDI 0167-4889(95)00163-8

such studies is to focus on a simple model system (e.g., *Aplysia*) which is devoid of the myriad biochemical processes occurring in an animal such as rat.

Even though *Aplysia* is a simpler model, a set of complementary neuronal cell lines would probably be ideal for such studies due the possibility of effective dissection of individual biochemical pathways in such cell lines. Our studies are focused on the serotonin 1A receptor which, as discussed in the following section, has been implicated in the trophic effect of serotonin. Of all known cell lines (including the ones derived from hippocampus and cortex), there is only one, the septal line SN 48, which expresses the 5-HT_{1A} receptor only after retinoic acid-mediated differentiation [12]. Therefore, for such studies, it is essential to engineer neuronal cell lines to stably express the serotonin 1A receptor, but thus far, it has been difficult to achieve stable expression of a neuronal receptor in a cell line of neuronal origin. We have achieved stable expression of the human serotonin 1A receptor in neuroblastoma cell lines obtained by hybridization of murine tumor line N18TG-2 with mouse hippocampal cells (HN2), Chinese hamster brain explant (NCB-20) or rat dorsal root ganglion cells (F-11).

A paradigm of stress termed here as 'nutrient deprivation', was designed to mimic the stages observed during neuronal maturation. The distinct stages observed in vivo are (1) proliferation, (2) migration, (3) differentiation and (4) apoptosis. During the process of differentiation which follows proliferation and migration, extensive apoptosis is also observed. This is in part due to the limited supply of protective proteins such as the region-specific trophic factors (e.g., BDNF or NGF) or other ubiquitous proteins such as bcl-2 or the insufficient supply of molecules (e.g., cAMP) which provide the appropriate signals required for neuronal differentiation [13]. A close morphological similarity to this process of maturation is observed in neuron-derived cell lines HN2, NCB-20, and F-11 during nutrient deprivation or continuous culture without feeding. The cells proliferate rapidly at the initial stage, move about on the dish to form appropriate contacts and clusters and close to confluency, show a high degree of apoptosis which continues for several days. This is marked by the clear morphological features of shrinkage in size and disintegration of the nucleus into smaller apoptotic bodies. If the medium is supplemented with appropriate differentiation-inducing agents (e.g., retinoic acid or dibutyryl cAMP), a significant proportion of the cells survive and undergo differentiation to form neuritic processes, growth cone-like structures and, upon co-culturing with myocytes, make synaptic contacts as reported earlier by others [14]. This is particularly similar to the in vivo situation where the committed neurons survive and differentiate whereas the uncommitted neurons which have not received a signal for differentiation undergo apoptosis. It has also been shown in other studies that the serotonin 1A receptor expression is dramatically increased between embryonic days 14–16 in rat brain [15]. This is also the stage at which the maximum loss of neurons takes place following migration. Thus in this study, Hillion and co-workers have suggested that serotonin binding to the serotonin 1A receptor has a trophic effect on maturing neurons and signal transduction through this pathway is enhanced by augmentation of 5-HT_{1A}-R expression to prevent neuronal loss [15]. In our present study, we have engineered the model cell lines described above (HN2, NCB-20 and F-11) in order to obtain neuronal clones stably expressing the serotonin 1A receptor. Following this, we have used such transfected neuron-derived cell lines to test the regulation of serotonin 1A receptor gene expression during the process of proliferation, maturation and loss of neuronal cells in a tissue culture model.

Interneuronal contacts are pivotal in the survival of neurons and the possibility of such contacts is determined by cell surface molecules such as gangliosides. Therefore, in the present study, our objective was also to determine if the stably expressed 5-HT_{1A} receptor had any effect on the membrane lipids which are expressed by the neuronal cells. It was already known from earlier studies that agonist binding to certain heptahelical membrane receptors, such as the 5-HT_2 or the bradykinin receptors, results in the stimulation of the effector phospholipase C, thereby causing increased formation of the intracellular lipid, IP_3. However, there is no report indicating coupling of a receptor to synthetic enzymes such as the glycosyltransferases which also result in an increase in the formation of a membrane lipid. The purpose of this study was to use engineered neuronal cells as a model to understand the regulation of the 5-HT_{1A} receptor as well as its role in the synthesis of important cell surface marker lipids such as gangliosides.

2. Materials and methods

The G-21 clone in pBCI vector was a generous gift from Dr. John Raymond and Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). Densitometry was carried out using a Microscan 1000 image analyzer and TRI Scan Programs from Technology Resources, Inc., Nashville, TN.

Transfection and preparation of clonal cell lines stably expressing the 5-HT_{1A} receptor: In each batch, 10^7 cells were electroporated with a mixture of *Pvu*I-linearized pBCI-G-21 (10 μg), pCH110 (5 μg) (encoding the enzyme, β-galactosidase, which was used to determine transfection efficiency), pSVneo (4 μg) and 500 μg salmon sperm DNA and following selection in the presence of 400 μg/ml Geneticin, clones were screened for highest [^{3}H]8-OH-DPAT binding activity. After selecting the best clones, plates of cells which were initially seeded at 5×10^5 per 10-cm plate were harvested at day 11 for each passage and washed once with a cold (4°C) swelling buffer (10 mM

Tris-HCl, pH 7.4, 5 mM EGTA) and then homogenized in the same buffer using a Potter-Elvehjem homogenizer in ice. The lysate thus obtained was centrifuged at 300 000 × *g* for 5 min, the supernatant discarded and the pellet washed once with the same buffer and then resuspended in buffer A_1 (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) by Potter-Elvehjem homogenization and stored at −80°C until assay. An aliquot (~ 1/10 th of the total number of cells) of each cell pellet was transferred to a microfuge tube pelleted at setting '7' at 4°C, washed once with PBS and then stored in aliquots at −80°C. Transfection efficiency was determined by β-galactosidase staining with X-gal and an aliquot of cells 72 h after transfection, and at the same time the remaining portion of the transfected cells was subjected to Geneticin selection.

Tissue culture and collection of samples during nutrient deprivation: Cells were seeded in DME-M with 10% fetal calf serum and 200 μg/ml Geneticin at 2.5×10^5 per 10-cm plate. Following seeding, the medium was not replaced during the entire time course. Each time point was an average of binding activities obtained from 2–3 independent plates and each assay was carried out in duplicate. While harvesting cells on specific days, even the floating cells were collected and included in all assays. Cell viability was determined by trypan blue exclusion with an aliquot of this cell suspension. Two aliquots of cells (~ 15% each) from each plate were removed, separated by centrifugation at 100 × *g*, washed once with 100 mM phosphate-buffered saline (pH 7.4), and then stored frozen at −80°C for hexosaminidase assay (see Discussion) and Northern blot analysis. The major portion of cells was separated by centrifugation and used for membrane preparation and the membranes stored at −80°C until [^{3}H]8-OH-DPAT binding analysis. Total RNA [16] was resolved on a 1.2% agarose gel, transferred to nitrocellulose membranes and probed as discussed below.

Binding assays: Binding assays were carried out in duplicate using 150 μg of membrane protein in a total volume of 1 ml in the presence of 5 nM [^{3}H]8-OH-DPAT at 37°C for 3 min (K_d ~ 1–2 nM) according to our earlier report [17]. Non-specific binding was determined in the presence of 10 μM serotonin.

RNA isolation and Northern blot analysis: Total RNA was isolated typically from $1–5 \times 10^6$ cells by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, Ref. [16]). RNA thus prepared was stored under ethanol at −80°C until use.

Northern blots were obtained by using 8–16 μg of total RNA. The G-21 DNA probe was released from G-21pBCI by digestion with *Hin*dIII and *Bam*HI, resolved by 1.2% low melting agarose gel electrophoresis, gel slice containing the G-21 sequence was melted at 65°C, diluted and an aliquot containing 50 ng of DNA labeled with α[^{32}P]dCTP by random priming in the presence of the Klenow fragment (using the Oligonucleotide Labeling Kit, Pharmacia, Alameda, CA). Prehybridization and hybridization were carried out in 25 mM potassium phosphate (pH 7.4), 5 × SSC, 5 × Denhardt's solution, 50 μg/ml salmon sperm DNA, 50% formamide and 10% dextran sulfate. Prehybridized blots were hybridized overnight at 42°C with radiolabeled G-21 probe. Following this, blots were washed twice with 1 × SSC containing 0.1% SDS at 42°C and then twice with 0.1 × SSC plus 0.1% SDS at 60°C. Autoradiography was carried out at −80°C with enhancing screens.

After probing with the G-21 sequence, the blots were stripped by incubation at 68°C in 1 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% sodium dodecylsulfate and then prehybridized overnight. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin probes were released from pGEM by digesting with *Pst*I. The excised DNA sequences were radiolabeled as discussed above and used for reprobing the blots.

Ganglioside analysis: Control and 5-HT_{1A}-R expressing cells were grown in DME-M (containing 10% fetal calf serum and Gentamicin) in 10-cm dishes for 8 days without medium replacement. On day 8, the medium was replaced with fresh medium (10 ml) and [^{3}H]palmitic acid (15 μCi/10-cm plate) in 3 μl ethanol was added to each plate followed by incubation in a 37°C incubator for 20 h for pulse labeling of cells. Next, the cells (4×10^7 per set) were harvested, washed with phosphate-buffered saline and the cell pellet was sonicated in 0.5 ml water. An aliquot (20 μl) was removed for protein estimation and 2 ml chloroform/methanol: 2: 1 (v/v) added to the remaining homogenate, followed by resonication and incubation at room temperature for 1 h. The suspension obtained was centrifuged at 100 × *g* and the upper layer supplemented with 2 ml chloroform, 0.5 ml methanol and 1.3 ml 0.74% KCl (aq), vortexed and the lower phase washed three times with 1.3 ml chloroform/methanol/0.74% KCl (aq): 3/48/47. The upper phases were pooled, evaporated, the residue redissolved in 1 ml chloroform/methanol/water (120: 60: 9, v/v) and loaded on a 5 × 0.25 cm Sephadex G-25 (fine) column pre-equilibrated with chloroform/methanol/water: 120:60:9 (v/v). Following elution with 10 ml of pre-equilibrating solvent, the pooled eluate was evaporated and the residue reconstituted in 500 μl chloroform/methanol (1:1, v/v). Aliquots corresponding to 2.4 mg cell protein were spotted on a 10 × 10 cm high-performance thin layer chromatography (HPTLC) plate, developed with chloroform/methanol/0.2% $CaCl_2$ (aq): 55:45:10, sprayed with ENHANCE and subjected to fluorography. Following this, the plates were dried and ganglioside bands visualized using resorcinol reagent.

The experiments involving 8-OH-DPAT treatment of cells were carried out after 20-h of pulse labeling of cells with [^{3}H]palmitate as discussed above and then the medium was supplemented with 10 μM 8-OH-DPAT. Following 8-OH-DPAT treatment, the medium was aspirated, the cells harvested and gangliosides extracted as discussed above.

3. Results

Passage-dependent increase in expression of the serotonin 1A receptor in transfected HN2 and NCB-20 cells: Transfection of the construct, pBCI-G-21, into HN2, NCB-20, F-11 and CHO cells was followed by selection of clones in the presence of 400 μg/ml Geneticin. Screening of clones by [^{3}H]8-OH-DPAT binding yielded four 5-HT_{1A}-R expressing clones, HN2-5, NCB-20T8, F-11T16 and T-CHO, which were used in the following experiments.

As discussed in the following section, the highest expression of the serotonin 1A receptor was observed in the neuronal cells at day 11 of continuous culture without feeding. Therefore, the highest [^{3}H]8-OH-DPAT binding obtained (most of the times at day 11) during continuous

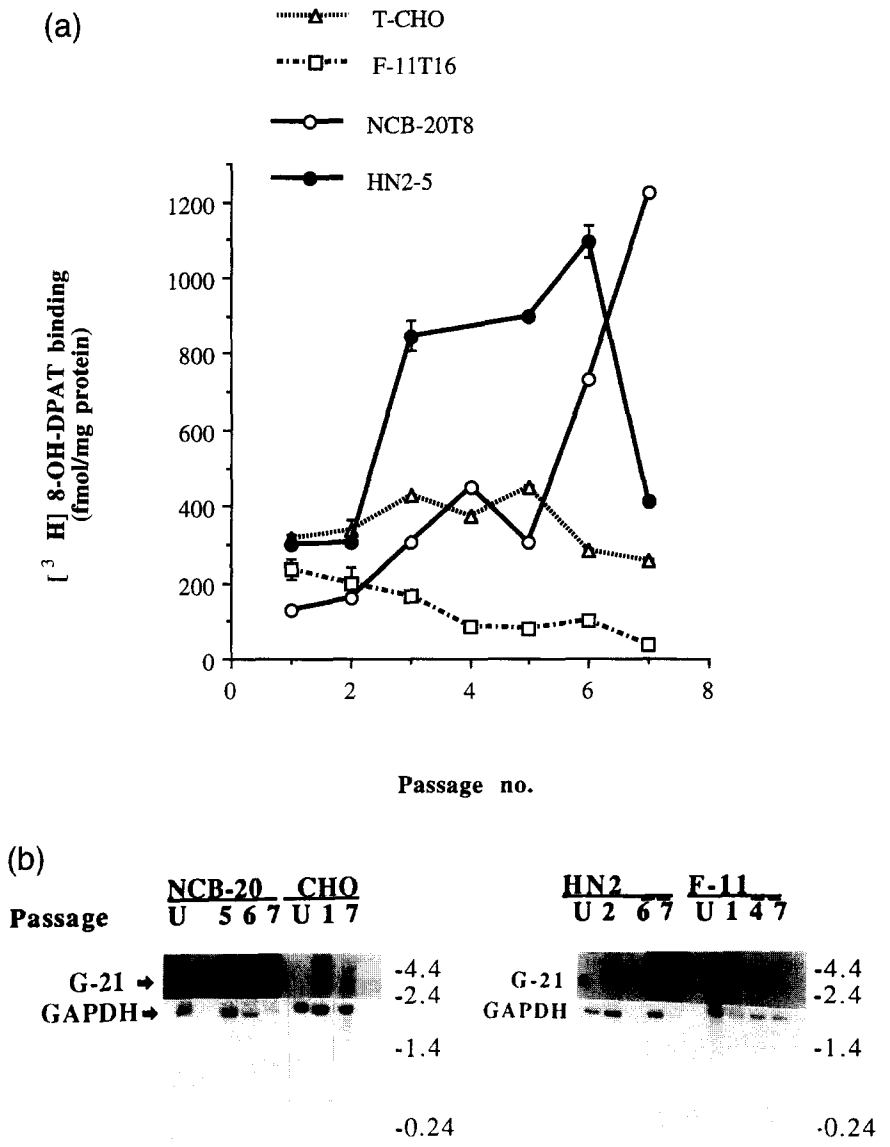


Fig. 1. (a) Passage dependence of [^{3}H]8-OH-DPAT binding activity. While a pronounced increase in activity was observed for HN2-5 (3-fold, passages 3–6) (●) and NCB-20T8 (8-fold, passages 6–10) (○) cells, the F-11T16 cells showed the highest activity at the first passage (after thawing) followed by a gradual decrease in the later passages (□). [^{3}H]8-OH-DPAT binding to the T-CHO cells remained virtually unchanged over several passages (Δ). (b) Northern blot analysis showing parallel increase in message levels in HN2 and NCB-20 cells. Cells from selected passages were harvested, total RNA isolated and subjected to Northern blot analysis (10 μg/lane). (Left panel) Untransfected NCB-20 (lane 1); NCB-20T8 (transfected), 5° (lane 2), 6° (lane 3), 7° (lane 4); untransfected CHO (lane 5); T-CHO, 1° (lane 6), 7° (lane 7); (Right) untransfected HN2 (lane 1); HN2-5, 2° (lane 2), 6° (lane 3), 7° (lane 4); untransfected F-11 (lane 5); F-11T16, 1° (lane 6), 4° (lane 7), 7° (lane 8). (°) = passage.

culture without feeding (as explained below) during every passage was considered here. During early passages (1 or 2), the density of stably expressed 5-HT_{1A} sites was not widely different (120-320 fmol/mg protein) in the four transfectants (HN2-5, NCB-20T8, F-11T16, and T-CHO). However, [^{3}H]8-OH-DPAT binding activity in the HN2-5 and NCB-20T8 cells increased 3-fold and 8-fold during passages 3–6 and 6–10, respectively (Fig. 1a). While a gradual, passage-dependent decrease in [^{3}H]8-OH-DPAT binding activity was observed in the F-11T16, there was virtually no change in agonist binding to the T-CHO cells over 7 passages. Message levels closely corresponded to the observed changes in [^{3}H]8-OH-DPAT binding activity in all the transfectants (Fig. 1b).

It may be argued that this passage-dependent increase in expression of the serotonin 1A receptor was due to further selection of a specific 5-HT_{1A}-R expressing clone with each passage. Evidence against this possibility is shown in our data. If this increase was due to further selection, then it would have been observed in all the 5-HT_{1A}-R expressing clones shown here. Instead, it occurred only in the HN2-5 and NCB-20T8 cells, whereas expression of the receptor was either unchanged or decreased in the other two transfected cell lines, T-CHO and F-11T16, even though Geneticin-selection occurred continuously in all the different clones. Therefore, it is difficult to clearly understand the physiological implication of this passage dependence of 5-HT_{1A}-R expression in certain neuronal cell types; nonetheless, it is an important piece of data which is required for further studies of signal transduction at optimum expression levels of the receptor. Most of our initial studies on the regulation of the serotonin 1A receptor and all of the studies on gangliosides were carried out during the high expression passages.

Dramatic increase in serotonin 1A receptor in the transfected neuronal cell lines during nutrient deprivation mediated decrease in cell viability: We have found that the stress of nutrient deprivation results in a time-dependent increase in 5-HT_{1A}-R expression in the transfected neural cells (NCB-20T8 and F-11T16), and this effect was diminished upon replacement of the old medium with fresh. Thus, in our initial experiments, we found that at day 7 of culture, unfed F-11T16 and NCB-20T8 cells contained 187% and 171% of [^{3}H]8-OH-DPAT binding, respectively, with respect to cells fed with fresh medium at day 5. In contrast, unfed, day-7 T-CHO cells contained only 105% of [^{3}H]8-OH-DPAT binding with respect to day-7 T-CHO cells fed at day 5. After studying the complete time course of this induction at three different passages, we found that expression of the receptor in the T-CHO cells (Fig. 2a) increased until confluence, with a subsequent decrease during the following phase of decreased cell viability (inset). In sharp contrast, and independent of passage number, in all the neural cells, expression of the receptor increased dramatically (day 10–12) during the phase of decreased cell viability (Fig. 2, upper panels, b,c,d). Virtually the same time profile is observed when cells are initially seeded at both 2.5×10^5 or 5×10^5 cells per 10-cm dish (only the profile obtained using 2.5×10^5 cells has been presented here).

Upon Northern blot analysis, it was revealed that this dramatic increase in serotonin 1A receptor expression was concomitant with a dramatic decrease in mRNA for the cytoskeletal protein β-actin as measured by hybridization to ^{32}P-labeled DNA probes for 5-HT_{1A}-R (G-21) and β-actin (Fig. 2, lower panel, i and ii). This decrease in β-actin message is an additional piece of evidence for the degradation of intracellular machinery during the phase of decreased cell viability as measured by Trypan blue exclusion.

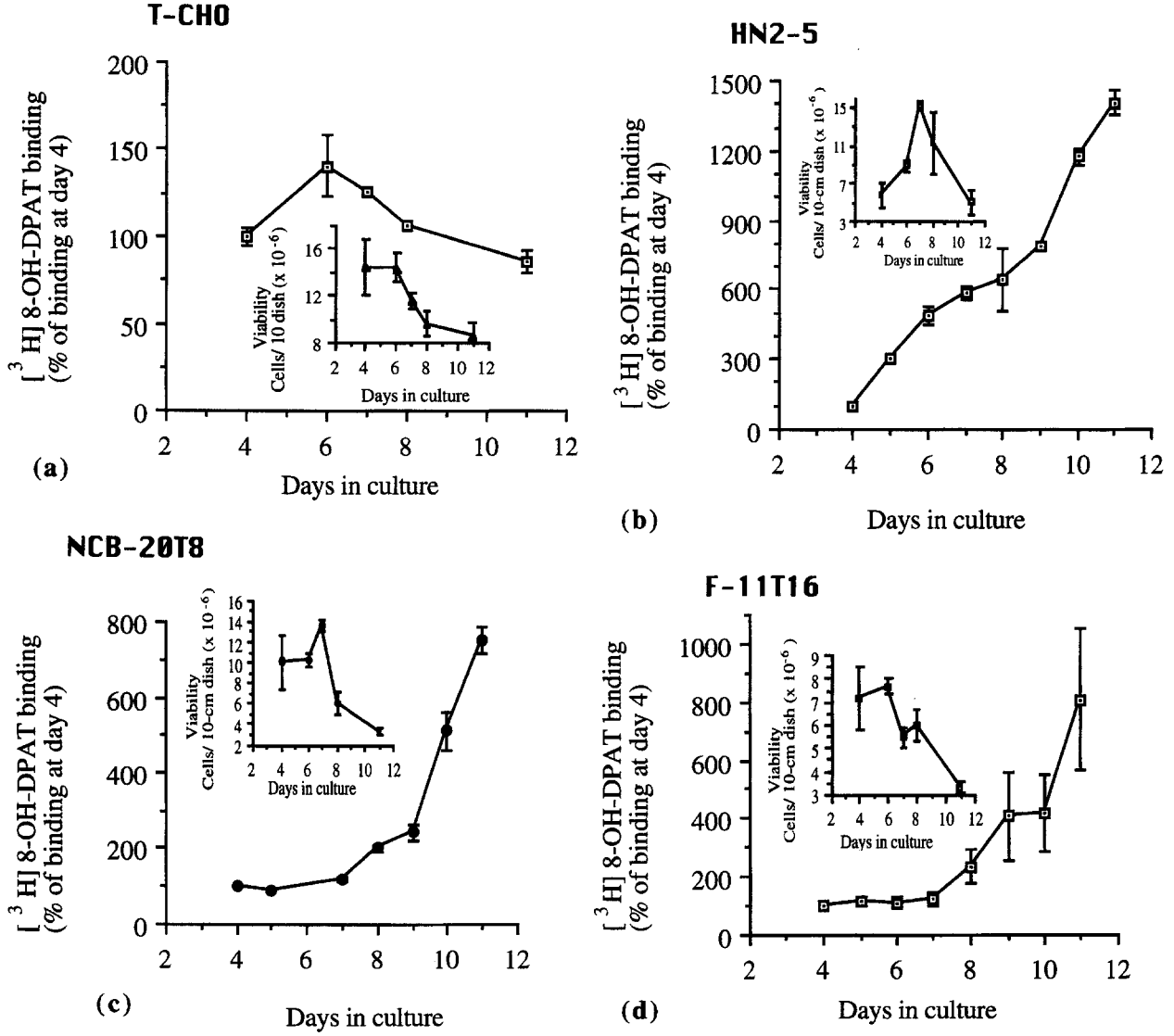
We also investigated the possibility of transcriptional activation during nutrient deprivation of an endogeneous 5-HT_{1A}-R gene present in the neuronal cell lines. Since the expression of the 5-HT_{1A}-R is the highest in the hippocampal neurons and virtually absent in the DRG, therefore, the chances of endogeneous expression of this receptor was the highest in the hippocampal HN2 cells. Therefore, the possibility of activation of an endogeneous 5-HT_{1A}-R gene was tested in the hippocampal HN2 cells. As shown in Fig. 2 (lower panel) (i), untransfected HN2 cells (lane 1) at day 11 of continuous culture without feeding showed no hybridization to the G-21 probe, thus proving the absence of transcriptional activation of an endogeneous 5-HT_{1A}-R gene in the untransfected HN2 cells. Therefore, the induction of the serotonin 1A receptor in the transfected clones was not due to a transcriptional activation of an endogeneous serotonin 1A receptor gene during nutrient deprivation.

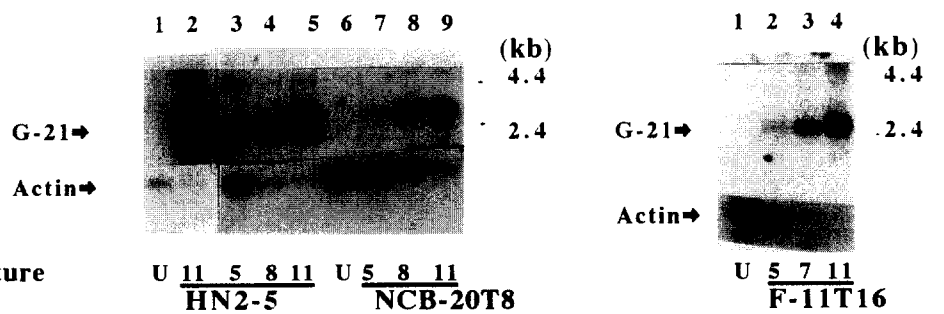
Differentiation and serum deprivation does not result in any significant increase in the expression of serotonin 1A receptors in the transfected neuronal cell lines: The maximum 5-HT_{1A}-R expression observed in the hybrid neuroblastoma cells was always at the late stationary phase. Therefore, it was possible that this increased expression was one of the changes observed during differentiation (e.g., acetylcholine esterase activity in the neuroblastoma cells, C-1300N18, is dramatically increased following restriction of cell growth by serum deprivation). Differentiation of HN2 cells with 5 μM all-trans retinoic acid (RA) in 1% serum containing DMEM resulted in extension of processes which were intensely stained with the neurofilament protein antibody, SMI 33, but not with GFAP antibody (unpublished observation). Differentiation of both NCB-20 and F-11 cells were carried out with 400 μM dibutyryl cAMP in serum-free DMEM. Parallel to this, the effect of a 4-day treatment with the mitogen, phorbol 12-myristate-13-acetate (PMA) (1 μM), on 5-HT_{1A}-R expression was also studied. However, following differentiation or mitogen treatment (Fig. 3) modest or no change in 5-HT_{1A}-R expression ([^{3}H]8-OH-DPAT binding) was ob-

served in HN2-5, NCB-20T8 and F-11T16 cells. This indicated that cell differentiation per se was not the cause of the induction of 5-HT_{1A}-R expression.

While a small increase of [^{3}H]8-OH-DPAT binding (a maximum of 1.5-fold, as compared to the 15-fold increase observed following nutrient deprivation) was observed in some experiments in differentiated HN2-5 cells, but in our later experiments with the F-11T16 and NCB-20T8 cells we always observed a small decrease in [^{3}H]8-OH-DPAT. The effect of such changes in the expression of the 5-HT_{1A} receptor on ganglioside synthesis remains to be studied.

Presence of the 5-HT_{1A} receptor resulted in a dramatic increase in complex ganglioside synthesis in the transfected F-11 cells: We carried out total lipid analysis of





U = untransfected, parent cell line.

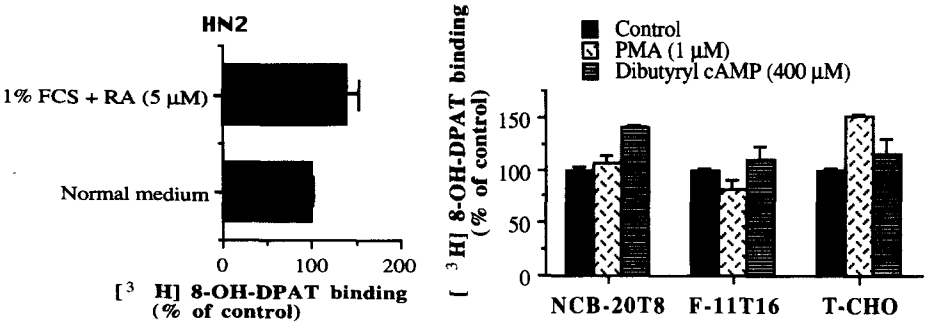


Fig. 3. Effect of cell differentiation on 5-HT_{1A}-R expression. (a) The HN2-5 cells were treated with retinoic acid (RA) (5 μM) in DMEM containing 1% fetal calf serum for 48 h. Differentiation of HN2-5 cells resulted in essentially no increase in [^{3}H]8-OH-DPAT expression over control HN2-5 cells maintained in normal growth medium (DME-M containing 10% fetal calf serum). (b) The other two cell lines, NCB-20T8 and F-11T16, underwent differentiation following a 4-day treatment with 400 μM dibutyryl cAMP in serum-free DMEM. No striking increase in [^{3}H]8-OH-DPAT binding activity was observed following differentiation or treatment with the mitogen, phorbol myristate acetate (PMA) (1 μM), for four days. Control cells were maintained in normal growth medium.

both transfected and untransfected cells at various stages of cell growth. A dramatic increase in [^{3}H]palmitate labeling (synthesis) of resorcinol-positive complex ganglioside bands comigrating with G_{D1a}, G_{D1b}, and G_{T1b} in the F-11 cells was observed following stable expression of the 5-HT_{1A}-R (Fig. 4a) at days 7/8 of culture. Also, a prominent increase in [^{3}H]palmitate labeling of ganglioside bands comigrating with minor (in the brain) gangliosides G_{M2} (3-fold by densitometry) and G_{D2} (between G_{D1a} and G_{D1b}) was observed in the F-11T16. Neither the untransfected F-11 cells nor a transfected and neomycin-resistant but 5-HT_{1A}-R-deficient clone (F-11T20), nor the other transfected cells (NCB-20T8 and T-CHO) showed this effect (Fig. 4b), thus confirming that this increase in radiolabeled gangliosides was strictly due to the presence of the 5-HT_{1A}-R, specifically in the dorsal root ganglion derived F-11 cells, and not an artifact of the transfection and selection procedure. Densitometric quantitation was carried out on those regions of lanes 2″, 3″ and 4″ which contained autoradiographic bands for the complex gangliosides, G_{D1a}, G_{D2}, G_{D1b} and G_{T1b} (indicated by asterisks). With an image analyzer, equal-sized rectangular blocks were used to include equivalent regions of lanes 2″ (contained the asterisked bands), 3″ (almost empty) and 4″ (almost empty) of Fig. 4 (regions indicated by bold, vertical lines drawn next to respective lanes, Fig. 4). In lanes 3″ and 4″, a band appeared just above the G_{D1a} doublet, this was probably due to G_{D3} which is normally present in significant quantities in the untransfected F-11 cells. Since there was no increase in this band in the 5-HT_{1A}-R expressing F-11T16 cells, it was not included in the quantitation of the [^{3}H]palmitate labeled complex ganglioside bands by densitometry. Upon densitometry using absorbance of transmitted light, a 20-fold increase in overall optical density of this region was observed in lane 2″ with respect to such regions in lanes 3″ and 4″ (all showing autoradiograms). This clearly indicates an increase in ganglioside synthesis in the presence of the 5-HT_{1A} receptor in the F-11T16 cells. In contrast to ganglioside synthesis, the overall complex ganglioside content of the F-11T16 cells was only 2- to 3-fold higher (by densitometry of the same regions of resorcinol stained bands in lanes 2, 3 and 4) than that in untransfected F-11 and the 5-HT_{1A}-R deficient F-11T20 cells. Therefore, the presence of the 5-HT_{1A}-R in the F-11 cells increased complex ganglioside synthesis without altering processing and metabolism of gangliosides.

In contrast to the increase in ganglioside synthesis in the dorsal root ganglion-derived F-11 cells, a 3-fold decrease in G_{D1a} synthesis was observed in the transfected

Fig. 2. Increased expression of the serotonin 1A receptor in neuroblastoma cells during nutrient deprivation and decreased cell viability. (Upper panel) [^{3}H]8-OH-DPAT binding (in fmol/mg protein, converted to fraction of binding at day 4) to cell membranes was measured at various stages of cell growth for the neuronal and non-neural transfectants. Cell viability was also recorded simultaneously (inset of each graph). The binding activity in the T-CHO cells increased until day 7 (the plates were confluent at day 4–5), following which a decrease was observed (a). In contrast, the [^{3}H]8-OH-DPAT binding increased dramatically after day 9 for the neuroblastoma transfectants, HN2-5 (b), NCB-20T8 (c), and F-11T16 (d) (plates were confluent at day 7 followed by a dramatic decrease in cell viability as shown in the inset). Maximum [^{3}H]8-OH-DPAT binding in pmol/mg protein were as follows: T-CHO (1.08), HN2-5 (1.08), NCB-20T8 (1.2), and F-11T16 (0.23). Binding activity profiles shown represent the mean (±S.D.) of 4–5 experiments carried out during both high expression passages as well as other passages. (Lower panel) Northern blot analysis showing a parallel increase in G-21 transcript levels in the transfected neuronal cell lines. (Left to right) HN2 cells, untransfected (day 11) lane 1 (16 μg total RNA); transfected (day 11) lane 2 (16 μg total RNA); time course of transfected HN2 cells (8 μg total RNA per lane): day 5 (lane 3), day 8 (lane 4); day 11 (lane 5). NCB-20 cells (8 μg total RNA per lane) : untransfected (lane 6); time course of transfected NCB-20 cells: day 5 (lane 7), day 8 (lane 8), and day 11 (lane 9). F-11 cells: untransfected (lane 1); transfected (F-11T16), day 5 (lane 2), day 7 (lane 3) and day 11 (lane 4). Note the inverse regulation of G-21 and actin mRNA bands during nutrient deprivation in the transfected neuroblastomas.

NCB-20 cells which contained no G_{D1b} or G_{T1b} before transfection. The 5-HT_{1A}-R-deficient clone NCB-20T10 showed higher [^{3}H]palmitate labeling of G_{D1a}, thus indicating that this modulation of ganglioside synthesis was due to the presence of the 5-HT_{1A}-R.

Many other ganglioside bands were labeled with [^{3}H]palmitate, however, as shown in lanes 2″ and 3″, there was no dramatic change in synthesis of other gangliosides moving slower than or equal to G_{M3}. Therefore, identification of each of those [^{3}H]palmitate labeled bands was not undertaken.

Such ganglioside analysis was also carried out on the transfected cells at both day 8 and day 11 of culture. However, essentially no change in ganglioside synthesis was observed during the phase of high receptor expression (day 11) (data not shown here). This was probably due to

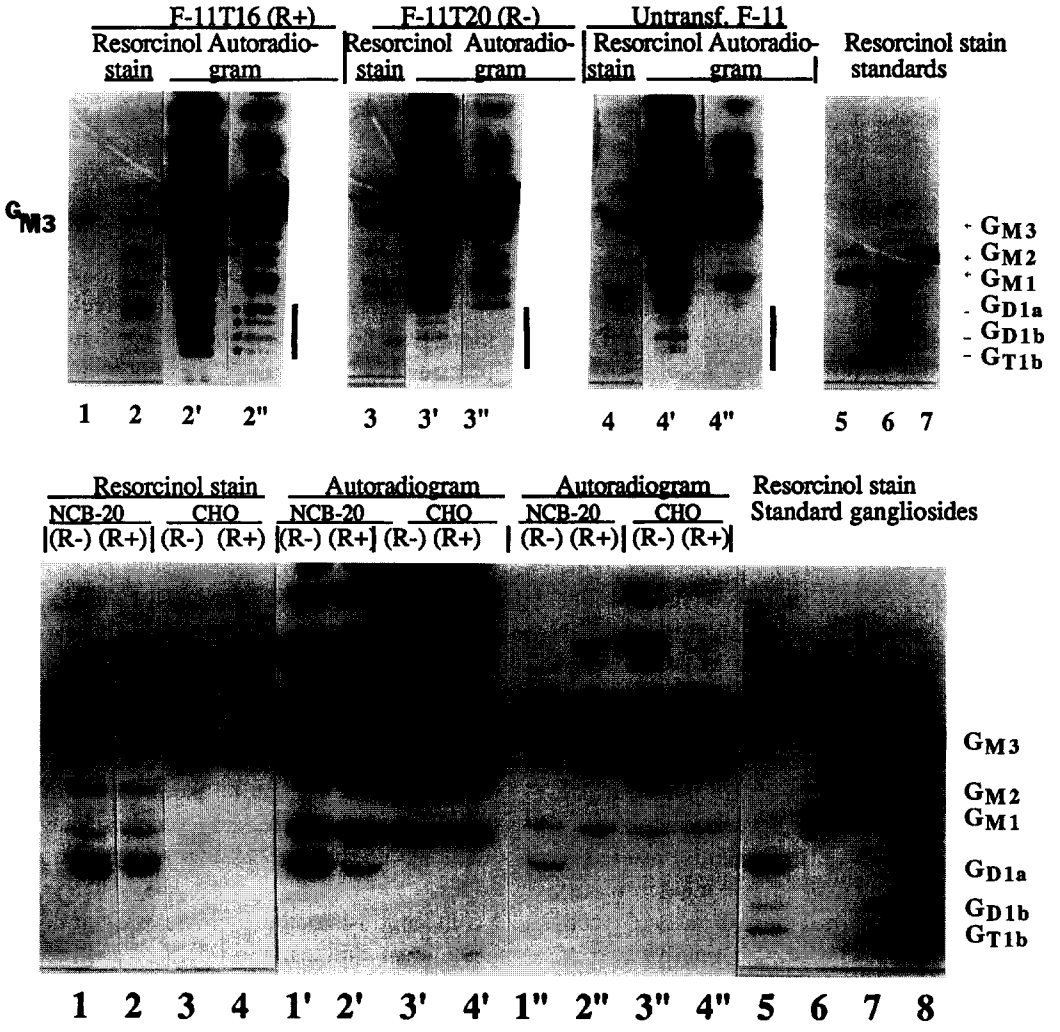


Fig. 4. Increase in [^{3}H]palmitate labeling of complex gangliosides in the F-11 cells in the presence of the stably expressed 5-HT_{1A}-R. Transfected cells stably expressing (R +) the 5-HT_{1A}-R (F-11T16, NCB-20T8, and T-CHO), transfected cells (Geneticin-resistant) not expressing (R −) the 5-HT_{1A}-R (F-11T20, NCB-20T10, and CHO-T3), and untransfected cells (F-11) were labeled for 20 h (at day 8) with [^{3}H]palmitic acid (15 μCi/10-cm plate) followed by ganglioside extraction and HPTLC analysis. (a) HPTLC profiles of resorcinol-stained lipids, G_{M3} (lane 1), F-11T16 lipids (lane 2), F-11T-20 (lane 3), untransfected F-11 lipids (lane 4), standard gangliosides, G_{M1} (lane 5), G_{D1a}, G_{D1b}, G_{T1b} (lane 6), G_{M2} (lane 7). The plate was subjected to fluorography for one week (autoradiograms) (lanes 2′, 3′, and 4′) and two days (lanes 2″, 3″, and 4″). Note the dramatic increase (20-fold by densitometry) in pulse labeling (synthesis) of ganglioside bands comigrating with G_{D1a}, doublet, G_{D1b}, G_{T1b}, and G_{M2} (indicated by asterisks) in the F-11T16 lipids (2′ and 2″) as compared to lipids obtained from F-11T20 (3′ and 3″) and F-11 (4′ and 4″) cells. Lane 2′ should be compared with lanes 3′ and 4′; lane 2″ should be compared with lanes 3″ and 4″; lane 2 should be compared with lanes 3 and 4. (b) A similar experiment was carried out with NCB-20T8 (R +), NCB-20T10 (Geneticin resistant but R −), T-CHO (R +), and CHO-T3 (R − but Geneticin-resistant). Resorcinol-stained lipids: gangliosides obtained from NCB-20T10 (lane 1), NCB-20T8 (lane 2), CHO-T3 (lane 3), T-CHO (lane 4); G_{D1a}, G_{D1b}, G_{T1b} (lane 5), G_{M1} (lane 6), G_{M2} (lane 7), and G_{M3} (lane 8). The HPTLC plate was exposed to an X-ray film for two days (lanes 1′, 2′, 3′, and 4′) and four hours (lanes 1″, 2″, 3″, and 4″). As compared to [^{3}H]palmitate labeling of resorcinol-positive bands of the Geneticin-resistant control clones (NCB-20T10, lanes 1′, 1″, and CHO-T3, lanes 3′, 3″), a decrease in pulse labeling of a ganglioside band comigrating with G_{D1a} (5-fold decrease) (by densitometry) was observed in the 5-HT_{1A}-R expressing NCB-20T8 (lanes 2′ and 2″).

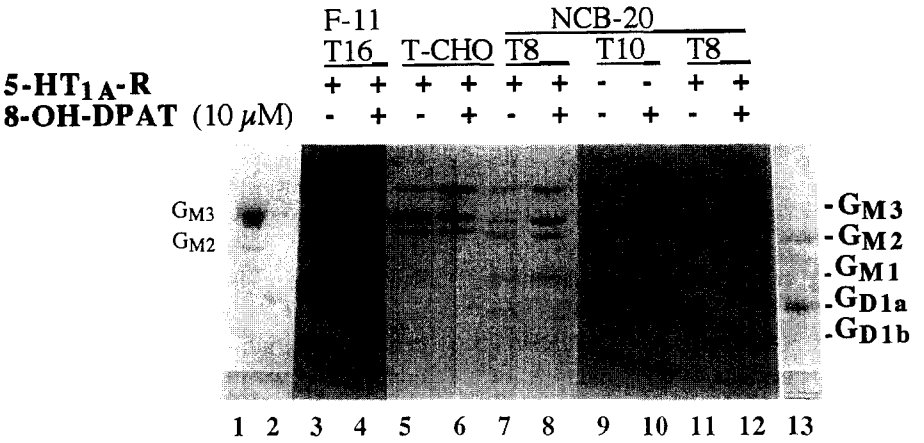
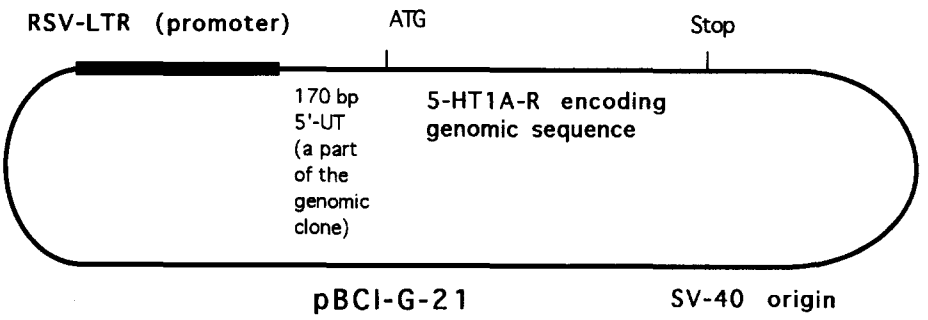


Fig. 5. Increase in a [^{3}H]palmitate labeled ganglioside band comigrating with G_{M3} in the NCB-20T8 cells following a 2-h treatment with 10 μM 8-OH-DPAT. A mixture of standard gangliosides, G_{M3}, G_{M2}, G_{M1} and G_{D1b} (lane 1), G_{D1a} (lane 2); without 8-OH-DPAT treatment: (autoradiograms) F-11T16 (R +) (lane 3), T-CHO (R +) (lane 5), NCB-20T8 (R +) (lane 7 and 11) and NCB-20T10 (R −) (lane 9); after 8-OH-DPAT treatment: F-11T16 (lane 4), T-CHO (lane 6), NCB-20T8 (R +) (lanes 8 and 12), and NCB-20T10 (R −) (lane 10). Note the pronounced increase in [^{3}H]palmitate labeling of a ganglioside band comigrating with standard G_{M3} after 8-OH-DPAT treatment of NCB-20T8 (R +) cells (compare lane 8 with lane 7) (3-fold by densitometry); standard ganglioside mixture containing G_{M2}, G_{D1a} and GD_{1b} (lane 13).

the impairment of many biochemical pathways including the cascades operative in the Golgi apparatus during the phase of decreased cell viability at days 10/11.

Selective 5-HT$_{1A}$ agonist (8-OH-DPAT) treatment resulted in an increase in a ganglioside comigrating with G_{M3} in the transfected NCB-20 cells: Agonist (1 μM 8-OH-DPAT) treatment for 2 h resulted in a 3-fold increase in [^{3}H]palmitate labeling (synthesis) of a ganglioside band comigrating with G_{M3} in the 5-HT$_{1A}$-R expressing (R +) clone NCB-20T8 (lane 7 and 8) but not in the other transfected cell lines (lanes 3–6) (Fig. 5). In the repeat experiment (lanes 9–12), the neomycin-resistant clone NCB-20T10 which did not express the 5-HT$_{1A}$-R (R −) showed no increase in [^{3}H]palmitate labeling of the ganglioside band comigrating with G_{M3} following 8-OH-DPAT treatment (lane 10 as compared to without 8-OH-



(a)

The 170-bp 5'-UT

```
1    TGCTCCTCGG AGATACCCTT CGCCGAAGCA GTAAGAACTT CCTGCTTGGG
51   TCTCTGCATT CCCTTCCTCC GAAACTTCCC AGGAGAAGGG CGGAAGACCC
101  CAGGGGAAGG GGCGAGGCGA ATCTTCGCGC TGCTTTTTCTTCCCTCCCCC
151  TTCCCGCGCC GGGCGCGCAG GCATG
```

(b)

Fig. 6. Sequence characteristics of the 1.7-kb genomic clone G-21. (a) The 5-HT$_{1A}$-R gene is intronless, like genes for many other heptahelical receptors, including the β_2 adrenergic receptor. Along with the coding sequence of the gene, the clone G-21 also contained a 170-bp 5'-end untranslated sequence. (b) The 170-bp untranslated genomic sequence of G-21 contains three copies of the heat shock element (HSE) (underlined) and several SP1 (GGGCGG), AP2, NFkB sites and a CAAT box. It is only a 3'-end portion of the 500-bp natural promoter for the human 5-HT$_{1A}$-R which contains no TATA box.

DPAT treatment, lane 9). In contrast, the 5-HT_{1A}-R expressing (R +) clone NCB-20T8 again showed an increase in labeling of this ganglioside band (with 8-OH-DPAT, lane 12; without 8-OH-DPAT, lane 11). The plates were subjected to fluorography for four days. The cells were harvested and gangliosides extracted and analyzed as described earlier. An unidentified lipid band moving slightly slower than G_{M3} (between G_{M2} and G_{M3}) was strongly labeled with [^{3}H]palmitate but not with resorcinol. While a clear separation of this band was achieved in the first experiment (its intensity was not affected by 8-OH-DPAT treatment), in the second experiment, some smearing of this band was observed in the 8-OH-DPAT-treated lane. However, due to the lack of resorcinol staining, this non-ganglioside (therefore it was not a slower-moving molecular species of G_{M3}) band was used as an internal control for loading of the lanes on the HPTLC plate.

4. Discussion

It was only in the neural cells that we observed a correlation between stress due to nutrient deprivation (which causes degeneration and eventually, cell death) and increase in serotonin 5-HT_{1A} receptor expression. Since a parallel increase in the G-21 transcript was also observed, this induction could be due to a specific effect of transcription factors and other elements, present only in the neuroblastoma cells, on the Rous sarcoma virus (RSV) promoter which drives expression of the 5-HT_{1A}-R-encoding G-21 DNA sequence. If this promoter (instead of the G-21 DNA sequence itself) was indeed responsible for the cell type-specific regulation of the 5-HT_{1A}-R, then our results demonstrate a special feature of the RSV promoter which has long been used for expressing various cDNA constructs. Alternatively, the intronless genomic sequence G-21 (Fig. 6), which contains a 170-bp untranslated 5′-end sequence (5′-UT) where several enhancer sequences (e.g., HSE, AP2 etc.) can be identified [15], could contain an enhancer sequence suitable for binding to transcription factors which are activated under nutrient deprivation. In a separate study, we have shown that the 5′-UT can result in an induction of the expression of chloramphenicol acetyltransferase (CAT) gene under nutrient deprivation (data not shown here). Thus, cell type-specific, increased expression of 5-HT_{1A}-R is due to a stress-sensitive enhancer element present in the 5′ untranslated region of the genomic clone G-21 (Fig. 6) used in our transfection experiments.

In other studies, we have already shown that our neuronal models (transfected F-11, NCB-20 and HN2 cells) can be used to demonstrate that agonist treatment of the serotonin 1A receptor causes inhibition of intracellular Ca^{2+} by either inhibition of N-type Ca^{2+} channels or by the inhibition of IP_3 formation [18]. This in turn could cause inhibition of Ca^{2+}-mediated excitotoxicity as observed in neurons during various types of stress. In view of such observations, data presented here suggest that the serotonin 1A receptor could be a protective protein, expression of which is dramatically up-regulated as a final attempt of the cells to regain normal function.

The second striking observation is that the serotonin 1A receptor, apart from its action in agonist binding and signal transduction, is found to play a modulatory role in the synthesis (measured by pulse labeling with [^{3}H]palmitic acid) of complex gangliosides in the DRG-derived F-11 cells and an inhibitory role in labeling of a ganglioside comigrating with G_{D1a} in the CNS-derived NCB-20 cells. Since membrane lipid association of the 5-HT_{1A} receptor has been shown in earlier studies [17], this observation has opened up further rigorous studies involving the measurement of glycosyltransferase activity in the various 5-HT_{1A}-R expressing clones. Our ongoing studies will eventually decide if this increased/decreased complex ganglioside synthesis is due to the action of the 5-HT_{1A}-R as an activator/inhibitor for glycosyltransferases in the cell lines reported here.

Comparison of resorcinol staining of ganglioside bands obtained from both 5-HT_{1A}-R expressing (F-11T16) and non-expressing (F-11T20 and untransfected F-11) cells, showed an increase (2- to 3-fold by densitometry) in complex gangliosides in the F-11T16 cells (Fig. 4, lane 2, as compared to lanes 3 and 4), however, a more dramatic increase (> 20-fold) was observed in [^{3}H]palmitate labeling of the same complex ganglioside bands (Fig. 4, lane 2″, compared to lanes 3″ and 4″). This indicated that ganglioside synthesis, rather than accumulation (due to a decrease in metabolism), was increased dramatically in the presence of the serotonin 1A receptor. Shorter exposures (for fluorography) revealed that there was no increase in gangliosides comigrating with G_{M1} or G_{M3} (Fig. 4a, lane 2″). Therefore, the likely candidate enzymes which could be positively regulated in order to cause this increase in specific gangliosides are N-acetylgalactosaminyltransferase, GalNAcT-1 (for elevated G_{M2} synthesis) [19], and the sialytransferases, SAT-2 (for G_{D3} and G_{D1b} synthesis) and SAT-4 (for G_{D1a} synthesis) [19]. β-Hexosaminidase assay carried out with extracts from the transfected cell lines (both 5-HT_{1A}-R + , NCB-20T8, and 5-HT_{1A}-R − , NCB-20T10, clones) showed essentially the same levels of endogeneous β-hexosaminidase B. Therefore, a down-regulation of the less specific, degradative enzymes, hexosaminidases and neuraminidases, which would result in an increase in many more gangliosides (including G_{M1} and G_{M3}), is unlikely in the present situation. This increased ganglioside synthesis in the F-11T16 was observed in three independent experiments each of which showed the most pronounced increase in a resorcinol-stained doublet corresponding to G_{D1a}, thus suggesting an increase in or stimulation of SAT-4. Intriguingly, the NCB-20 cells showed a single resorcinol positive band for G_{D1a}, and there was a 5-fold decrease in [^{3}H]palmitate

labeling of this lipid band in the 5-HT_{1A}-R expressing NCB-20T8 cells (Fig. 4b). Thus, the presence of the 5-HT_{1A}-R could also result in a down-regulation of specific gangliosides in a cell type-specific manner.

While receptor-mediated control of enzymes such as adenylate cyclase, phospholipase C and D as well as of ion channels and phosphate uptake have been the focus of several studies, the role of a glycosyltransferase as an effector in such signal transduction processes has seldom been addressed before. We have observed that a two-hour treatment (which can induce only early protein synthesis) of the NCB-20T8 cells with the 5-HT_{1A} agonist, 8-OH-DPAT (10 μM), results in a pronounced increase (3-fold by densitometry) in a ganglioside band corresponding to G_{M3} (Fig. 5). While this increase in ganglioside synthesis is not observed in the F-11T16 or T-CHO cells (Fig. 5), similar experiments are yet to be carried out with the HN2-5 cells. The choice of two hours as the duration of 8-OH-DPAT treatment was based on the fact that substrate conversion by the G_{M1}- and Lc_2Cer- sialyltransferases which synthesize G_{D1a}, G_{D1b}, G_{T1b}, G_{D2} and G_{M3} (SAT-4, SAT-2 and SAT-1) is linear for up to 2–3 h [19].

Receptor-mediated increase in degradation of lipids (by activation of phospholipase C and acyl-CoA oxidase) [20,21] has been demonstrated before. As far as synthesis of lipids is concerned, it was previously shown that prolonged treatment (18 h) with serotonin causes a 3-fold increase in [^{3}H]Gal incorporation into the gangliosides, G_{M2}, G_{M1} and G_{D1a}, in the untransfected NCB-20 cells [22]. This was explained as a secondary effect to cAMP elevation mediated by a serotonin receptor with $K_d \sim 180$ nM [23] for serotonin binding (as compared to a K_d of 5–10 nM for the 5-HT_{1A}-R) (probably the 5-HT_4 or 5-HT_7 receptor) [24]. Since [^{3}H]palmitate labeling of the ganglioside comigrating with G_{M3}, as observed in the present case, was increased within 2 h (only early protein synthesis is turned on in 2 h), this is the first evidence for a direct effector-like activity shown by a member (probably SAT-1, which is responsible for G_{M3} synthesis) of the family of synthetic enzymes, glycosyltransferases, which also glycosylate proteins and determine the final disposition and localization of these macromolecules in a cell. Such carbohydrate modifications may be the deciding step in the targeting of key enzymes (e.g., the various kinases) which regulate DNA synthesis. Thus our results are in agreement with earlier observations suggesting involvement of yet unidentified effector molecules in DNA synthesis mediated by the 5-HT_{1B}-G_i cascade in smooth muscle cells [25].

In summary this report shows the first stable expression of the serotonin 1A receptor in neuronal cell lines. This first stable expression of the 5-HT_{1A}-R has shown that, unlike in the non-neural cells, expression of this receptor is strongly regulated in the neuronal cells and also that this membrane protein (5-HT_{1A}-R) exerts strong regulation on ganglioside synthesis.

Acknowledgements

Excellent technical assistance by Messrs. Brett A. Chromy and Michael J. Boyers is gratefully acknowledged here.

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